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13. ABSTRACT *(Maximum 200 words)*

Taxol is a microtubule poison that has been used successfully in refractory breast cancer. Apart from its well characterized anti-mitotic effects, Taxol shares with bacterial lipopolysaccharide (LPS) the capacity to elicit microtubule-independent, intracellular signaling in murine macrophages that activate kinase cascades that lead to expression of many genes. This IDEA grant proposed to test the ability of Taxol to up-regulate expression of two genes, *nm23* and *c-kit*, whose expression is down-regulated in advanced, metatstatic breast cancer. In addition, modulation of adrenomedulin (AM), as well as a panel of inflammatory genes, were examined in murine macrophages and/or breast cancer cells stimulated by LPS or Taxol. Using optimized conditions for the detection of mRNA species by reverse transcriptase polymerase chain reaction (RT-PCR), we have (1) nearly completed studies on the modulation of AM in in macrophages, (2) demonstrated differential modulation of nm23 and c-kit mRNA in in the murine breast cancer cell line, DA-3, and (3) demonstrated that both LPS and Taxol strongly up-regulate expression of a panel pro-inflammatory genes in the breast cancer cell line. We have been granted a no-cost extension to move forward with our analysis of other murine and human breast cancer cell lines as originally proposed.

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Stephanie Vogel 7/13/98

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INTRODUCTION

Taxol is the prototype of a new class of microtubule stabilizing agents that has generated great enthusiasm in the oncology community due to its favorable response rate in patients with aggressive, metastatic cancers, including breast cancer (1). In addition to its anti-mitotic effects on cells, which is mediated by its ability to bind to β-tubulin and prevent the microtubule depolymerization required for cell division, Taxol has also been shown in murine macrophages to mimic the action of bacterial lipopolysaccharide (LPS) to modulate gene expression and tyrosine phosphorylation of proteins (2). More recently, Haskill *et al.* (3) have found that Taxol also modulates inflammatory gene expression in certain primary ovarian tumor cells. For this reason, we hypothesized that Taxol may also exert its anti-tumor effects by modulating the expression of certain genes whose expression is dysregulated in breast cancer. We proposed in our original grant to analyze in both macrophages and breast cancer cells the effects of Taxol and LPS on the expression of two genes, *nm23* and *c-kit*, whose expression has been found to be down-regulated in breast cancer (4,5). Conversely, the gene that encodes adrenomedullin (AM) has been reported to be expressed by many cancer cells and AM has recently been implicated as an autocrine growth factor in malignant cells (6). For this reason, expression of the AM gene, and its modulation by Taxol and LPS, were also analyzed in macrophages and breast cancer cells. The main hypothesis to be tested is that Taxol and/or LPS may modulate expression of these genes not only in macrophages, but also in in breast cancer cells, where their expression is dysregulated. Such a modulation could result in restoration of normal homeostatic gene expression or the production of pro-inflammatory genes by the tumor that activate immune cell types to attack the tumor.

BODY

In our last progress report, we detailed our findings with respect to the (1) development of the capability to detect nm23, c-kit, and AM mRNA expression by RT-PCR technology, (2) confirmation of detection of these species in murine melanoma cell lines and murine macrophages (the RAW 264.7 or C3H/OuJ macrophages), (3) the failure of either LPS or Taxol to modulate nm-23 or c-kit significantly, under conditions where AM mRNA was strongly upregulated. Since submitting that annual report, we have completed a manuscript on the regulation of AM mRNA expression by LPS and Taxol and submitted it for consideration for publication. We have recently heard from the peer-reviewed journal, *Infection and* Immunity, that the paper is acceptable with revisions. The major additional piece of information that was requested by the reviewer was the inclusion of protein data that would confirm the strong up-regulation of AM gene expression at the level of mRNA. Using radioimmunoassay to detect AM secretion, we have demonstrated that both LPS and Taxol induce secretion of AM that parallels closely the results that were obtained at the level of mRNA. A copy of the revised manuscript is included in the Appendix of this annual report (Zaks-Zilberman *et al.*, "Induction of

Adrenomedullin Expression by Lipopolysaccharide and Taxol in Murine Macrophages"). The data presented in this manuscript was presented, in part, at the NIH Adrenomedullin Symposium that was held in September 1997. A copy of the abstract of the meeting is also included in the Appendix.

We have continued our studies, focusing on an analysis of gene expression in the murine breast cancer cell line, DA-3. As detailed in our previous annual report, we failed to observe significant modulation of nm-23 and c-kit mRNA by LPS or Taxol in macrophages and murine melanoma cell lines, in the face of strong modulation of AM expression in macrophages. Taking this information together with the recent findings of White *et al.* (7), that Taxol induced expression of the cytokine gene IL-1β, but not the chemokine gene, IL-8, in a murine breast cancer cell line, MCF-7, we increased our panel of candidate genes to include cytokine genes (e.g., IL-1β, GM-CSF, M-CSF, G-CSF) and chemokine genes (e.g., IP-10, MIP-2, JE, MIP-1β, MCP-5), which we had found in our previous studies to be inducible by both LPS and Taxol in murine macrophages. In general, the cytokines chosen for this analysis have been strongly implicated in the induction and amplification of inflammatory responses, and the chemokines, for their capacities to cause the influx of inflammatory cell types into the affected region.

The data from these studies are shown in Figures 1-4, with a Summary in Table I. Briefly, Figure 1 demonstrates that Taxol and LPS strongly induced the expression of GM-CSF mRNA, with optimal expression after only 1-2 hr in the murine breast cancer cell line, DA-3. This is an important finding because GM-CSF, which has been used experimentally in the treatment of a number of tumors, has been demonstrated to result in the differentiation of macrophages to a highly tumoricidal state *in vitro* (8) and, therefore, may increase host defenses against the tumor by increasing the tumoricidal capacity of macrophages within the tumor microenvironment. The chemokine IP-10 was strongly upregulated (>10-fold) by Taxol, and to a much lesser extent by LPS (2-3-fold), and optimal gene expression occurred later (>8 h) than was observed with GM-CSF. Figure 2 illustrates the effects of a second group of genes which we have identified as "moderately inducible" (3-8-fold) by both LPS and Taxol. These include the chemokine, MIP-2, and the cytokines, IL-1β and G-CSF. Among genes that exhibited "low inducibility" in the DA-3 cell line are nm-23(A), showing optimal induction with either LPS or Taxol of only 2-3-fold over basal levels (Figure 3). Included in this grouping also was LPS-induced IP-10 (Figure 1, right panel). Although we were able to detect basal levels of M-CSF mRNA in the DA-3 cell line, neither Taxol nor LPS modulated its expression. Finally, the following genes were neither expressed in DA-3 cells nor were they inducible following treatment with LPS or Taxol: JE, MCP-5, MIP-1β, AM, and c-kit. Thus, although Taxol and LPS were able to up-regulate significantly the expression of several critical cytokine and chemokine genes, they failed to upregulate expression of nm-23(A) or c-kit.

To evaluate the capacity of Taxol and LPS to induce gene expression in the DA-3 cells, a dose-response experiment for LPS and Taxol was next carried out. The data

in Figure 4 demonstrate that LPS and Taxol induce measureable increases in GM-CSF mRNA expression with as little as 0.01 ng/ml and 5 μM, respectively. These doses are at least comparable to those required to elicit gene expression in murine macrophages. We are beginning to measure protein secretion for those genes that are up-regulated to confirm the data that we have obtained at the level of steady-state mRNA.

CONCLUSIONS

During the first year of funding on this project, we have developed all of the molecular methodology required to evaluate the effects of Taxol and LPS on the specific expression of nm23, c-kit, and AM mRNA in murine macrophages and in breast cancer cell lines. In addition, differential expression of the three genes in control melanoma cells and in the macrophages was observed. Taxol and LPS up-regulated expression of AM, and to a lesser extent, c-kit and nm23, in macrophage cultures. We have now completed our analysis of the effects in macrophages of LPS and Taxol on genes that have been reported in the literature to be either repressed (e.g., nm23 or c-kit) or augmented (e.g., AM) in breast cancer cells.

During the second year of funding, we have continued our work as proposed initially, by moving from an analysis of gene expression in macrophages to an analysis of these genes, as well as others, in the murine breast cancer cell line, DA-3. Although we were unable to demonstrate modulation of c-kit mRNA in these cells, nm-23 mRNA was induced by both LPS and Taxol, albeit to a relatively low extent. However, our data strongly support the exciting hypothesis that Taxol and LPS induce in a murine breast cancer cell line a wide array of pro-inflammatory cytokine genes, as well as genes that encode chemokines, factors that are capable of attracting inflammatory cell types to the tumor site. Thus, it is likely that the remarkable efficacy of Taxol, as opposed to other agents (e.g., vinblastine or colchicine) that also target microtubules to elicit their anti-tumor actions, may be attributable to Taxol's additional ability to mount an inflammatory response against the tumor by causing the tumor to produce proinflammatory cytokines and chemokines that, in turn, recruit and activate inflammatory cell types, such as macrophages.

As of the end of the last reporting period, we had completed, in part or in entirety, Tasks 1a, b and Tasks 2a, b, and c. We have now moved forward to complete, in part or in entirety, the analysis of gene expression in the breast cancer cell line, DA-3 (Task 1c) and have initiated experiments to evaluate the potential synergy between LPS and Taxol with IFN-γ (Task 1d) and protein measurements to support the mRNA data (Task 1e). Because we had a delay in initial funding, that, in turn, led to a delay in hiring the research associate who is dedicated to this project, we requested a no-cost extention from the Army, which was approved. We plan to complete the remaining tasks (1f, for the detection of phosphorylated protein species in DA-3 cells, as well as to confirm our findings with DA-3 cells using another breast cancer cell line.) We anticipate finalizing these studies in the upcoming year.

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APPENDICES:

Table I

Figure legends

Figures 1, 2, 3, 4

Zaks-Zilberman *et al.*. Induction of Adrenomedullin expression by lipopolysaccharide and Taxol in murine macrophages. *Infection and Immunity* (accepted with revisions).

Zaks-Zilberman *et al.* Upregulation of murine adrenomedullin mRNA expression by lipopolysaccharide (LPS) and Taxol. Abstract, NIH Adrenomedullin Symposium, September 3-5, 1997.

Table I: Summary of Induction of Gene Expression in DA-3 Murine Breast Cancer Cells by LPS or Taxol

	GENE	Maximum Induction	n*
HIGHLY INDUCIBLE	GM-CSF	≥10-fold	5
	IP-10 (Taxol only)	>10-fold	2
MODERATELY INDUCIBLE	G-CSF	3-8-fold	4
	MIP-2		2
	IL-1β		4
LOW INDUCIBILITY	NM-23(A)	2-3-fold	4
	IP-10 (LPS)		2
NOT INDUCIBLE	M-CSF		2
NOT EXPRESSED OR MODULATED	JE		2
	MIP-1β		2
	AM		2
	c-kit		2
	MCP-5		2

*n represents the number of independent experiments from which the data in Figures 1-4 were calculated.

FIGURE LEGENDS

Figure 1. Kinetics of GM-CSF (left) and IP-10 (right) mRNA induction by Taxol (35 mM) and LPS (100 ng/ml) in the murine breast cancer cell line, DA-3. GM-CSF and IP-10 mRNA species were quantified by RT-PCR using 28 and 31 cycles of amplification, respectively, at annealing temperatures of 54° C and 55° C, respectively. The results, summarized in Table I, represent the arithmetic mean ± SEM of 5 and 2 separate experiments, respectively.

Figure 2. Kinetics of MIP-2 (left), IL-1β (center), and G-CSF (right) mRNA induction by Taxol (35 mM) and LPS (100 ng/ml) in the murine breast cancer cell line, DA-3, of MIP-2, IL-1β, and G-CSF mRNA species were quantified by RT-PCR using 33, 32, and 26 cycles of amplification, respectively, at annealing temperatures of 65° C, 54° C, and 57° C, respectively. The results, summarized in Table I, represent the arithmetic mean ± SEM of 2, 4, and 4 separate experiments, respectively.

Figure 3. Kinetics of NM-23(A) mRNA induction by Taxol (35 mM) and LPS (100 ng/ml) in the murine breast cancer cell line, DA-3. NM-23(A) mRNA was quantified by RT-PCR using 25 cycles of amplification at an annealing temperature of 50° C. The results, summarized in Table I, represent the arithmetic mean ± SEM of 4 separate experiments.

Figure 4. Dose-response analysis of GM-CSF mRNA induction by Taxol and LPS in the murine breast cancer cell line, DA-3. GM-CSF mRNA species were quantified by RT-PCR using 32 cycles of amplification at an annealing temperature of 54° C. The results, summarized in Table I, represent the arithmetic mean ± SEM of 2 separate experiments.

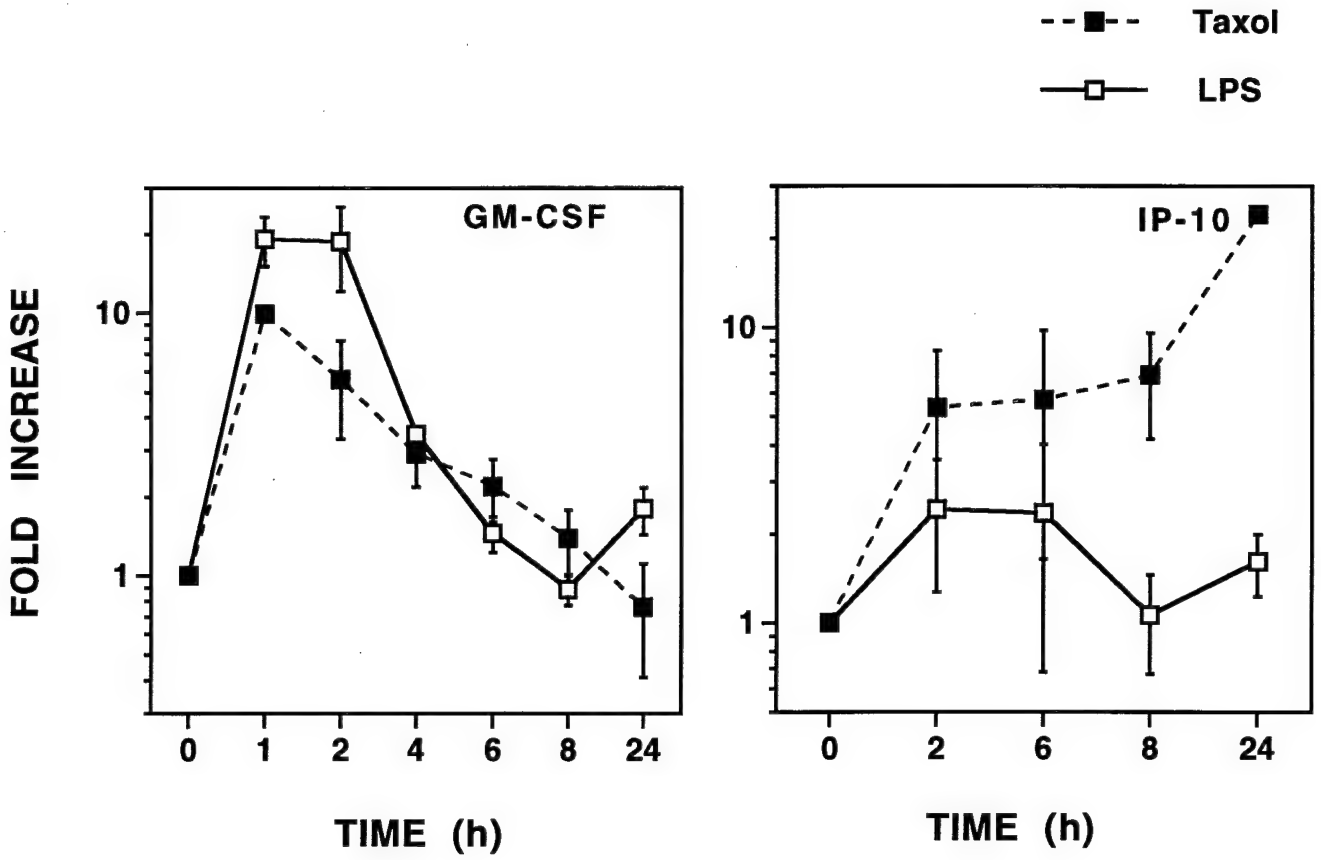

Taxol
LPS
GM-CSF
IP-10
FOLD INCREASE
10
1
0
1
2
4
6
8
24
TIME (h)
10
1
0
2
6
8
24
TIME (h)

FIGURE 1

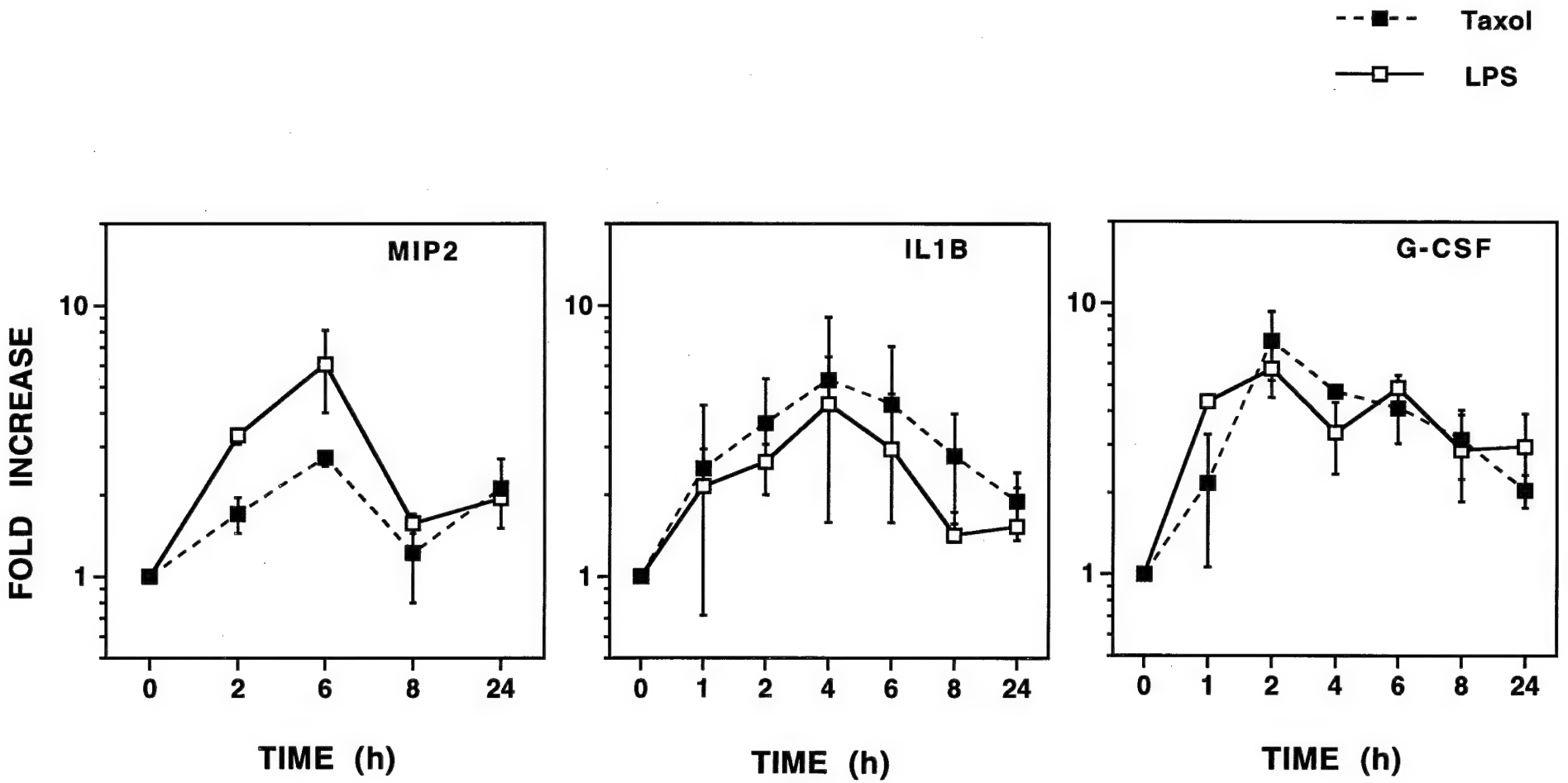

Taxol
LPS
MIP2
IL1B
G-CSF
FOLD INCREASE
10
1
0
2
6
8
24
0
1
2
4
6
8
24
TIME (h)

FIGURE 2

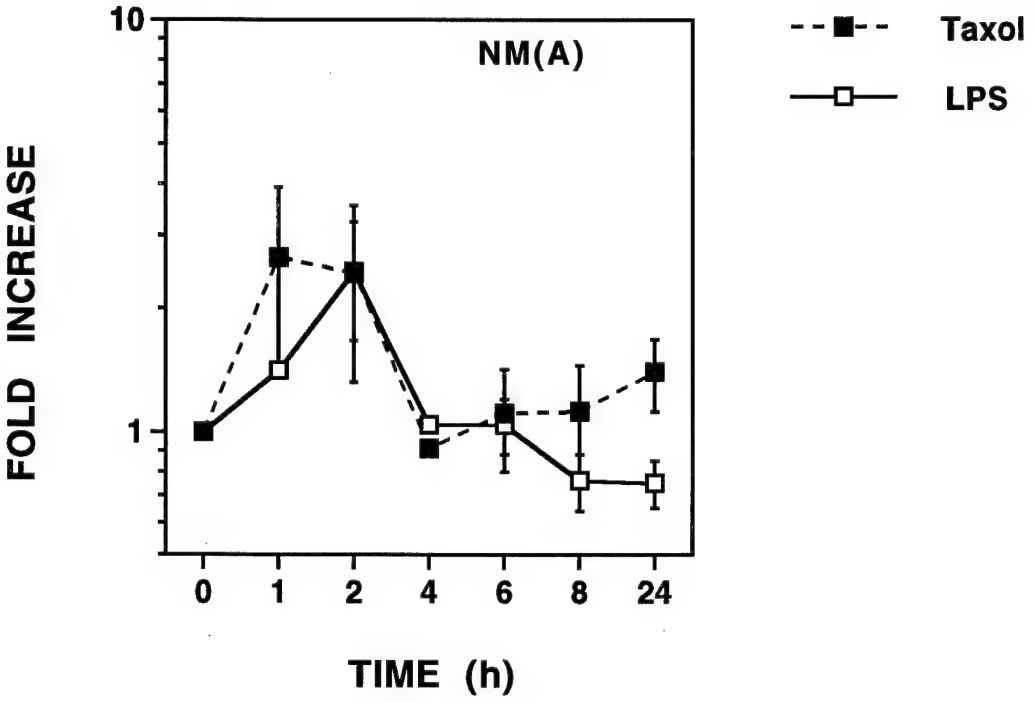


FIGURE 3

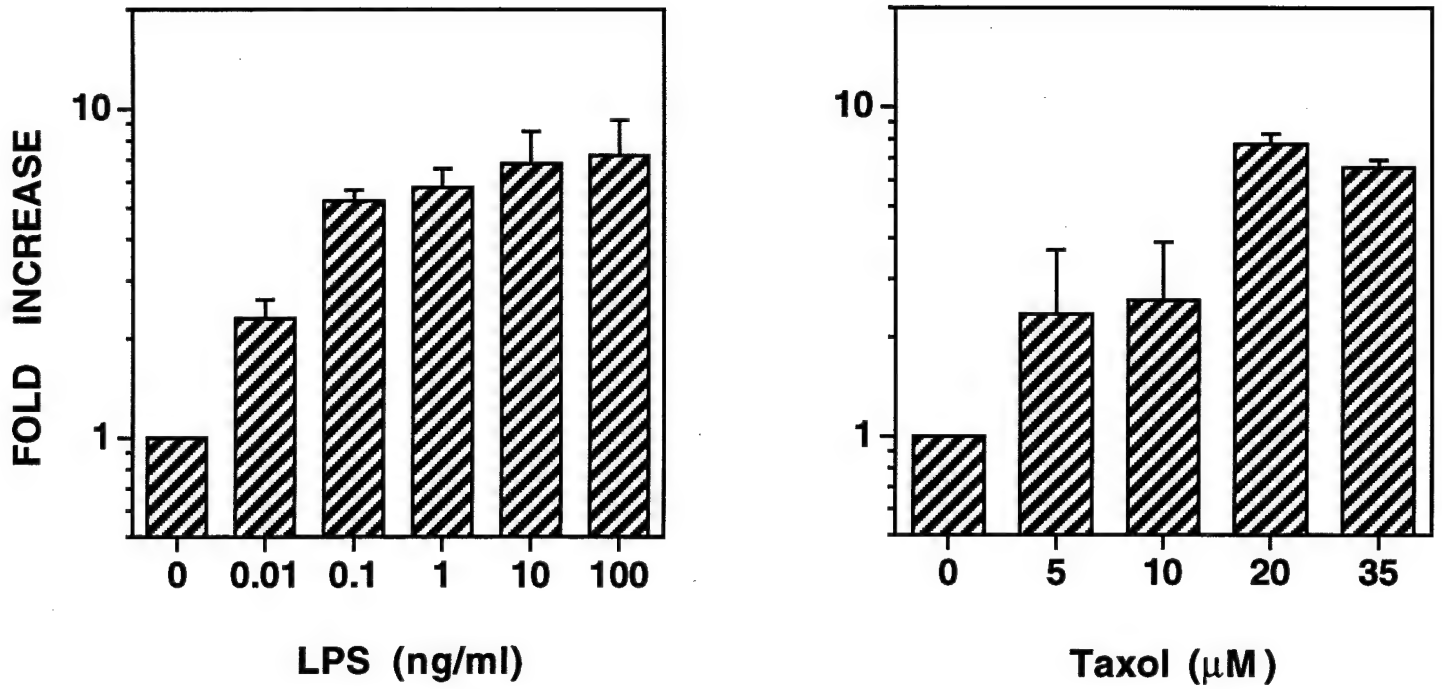
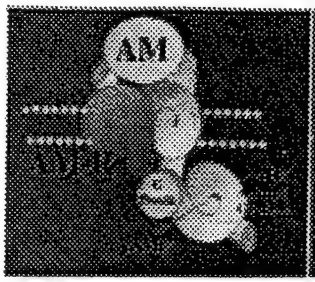

FOLD INCREASE
10
1
0
0.01
0.1
1
10
100
LPS (ng/ml)
10
1
0
5
10
20
35
Taxol (μM)

FIGURE 4

Symposium date: 9/3-5/97
The symposium will take place at the Natcher Conference Center in Bethesda, MD.



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Last name Zaks-Zilberman First name Meirav Middle initial

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UPREGULATION OF MURINE ADRENOMEDULLIN mRNA EXPRESSION BY LIPOPOLYSACCHARIDE (LPS) AND TAXOL.

Zaks-Zilberman M, Salkowski CA, Vogel SN.

Department of Microbiology and Immunology. Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.

Lipopolysaccharide (LPS) is a potent inflammatory stimulus derived from the outer membrane of Gram negative bacteria that has been implicated in septic shock. LPS stimulation of macrophages leads to a complex series of intracellular signaling events that culminate in the expression of a large number of inflammatory and anti-inflammatory genes. Recently, the antitumor agent, Taxol, was demonstrated to be an LPS-mimetic in murine macrophages. Since adrenomedullin (AM) is a potent vasorelaxant and since septic shock has been associated with vasorelaxation, we investigated the capacity of LPS and Taxol to induce AM in murine macrophages. When peritoneal exudate macrophages from C3H/OuJ mice were treated with protein-free, *E. coli* K235 LPS (100 ng/ml) or Taxol (35 μg/ml), a ~5-fold increase in AM steady-state mRNA levels was observed, peaking between 2 and 4 hours, and returning to baseline after 8 hours. While LPS-hyporesponsive C3H/HeJ macrophages failed to respond to protein-free LPS with an increase in AM steady-state mRNA levels, increased levels were observed after stimulation of these cells with a protein-rich (butanol-extracted) LPS preparation. In addition, increased AM mRNA was observed following treatment of either C3H/OuJ or C3H/HeJ macrophages with soluble *T.gondii* tachyzoite antigen (STAg) or the synthetic flavone analogue 5,6-dimethylxanthenone -4- acetic acid (5,6-MeXAA). Interferon-γ also stimulated C3H/OuJ macrophages to express increased AM mRNA levels. *In vivo*, LPS administration also led to augmented AM gene expression. Mice challenged with 25 μg LPS (i.p.) exhibited increased AM mRNA levels in lung, liver, and spleen, with the greatest increase (>50-fold) observed in the liver. These data indicate that AM represents yet an additional LPS-inducible gene product that may contribute to the induction of Gram negative septic shock through its action on the vascular system. (Supported by NIH AI-18797 and USAMRDC DAMD17-96-1-6258.)

Revised
7/18/98

Induction of Adrenomedullin mRNA and Protein by Lipopolysaccharide and Paclitaxel (Taxol™) in Murine Macrophages[1]

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Running Title: Modulation of Adrenomedullin in murine macrophages

FOOTNOTES

[1] This work was presented in part as a poster presentation at NCI Adrenomedullin Symposium, Sept. 3-5, 1997, Bethesda, MD.

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ABSTRACT

Lipopolysaccharide (LPS), a potent inflammatory stimulus derived from the outer membrane of Gram negative bacteria, has been implicated in septic shock. Plasma levels of adrenomedullin (AM), a potent vasorelaxant, are increased in septic shock and possibly contribute to the characteristic hypotension. As macrophages play a central role in the host response to LPS, we studied AM production by LPS-stimulated macrophages. When peritoneal exudate macrophages from C3H/OuJ mice were treated with protein-free LPS (100 ng/ml) or the LPS-mimetic, Taxol (35 μM), a ~10-fold increase in AM steady-state mRNA levels was observed, which peaked between 2 and 4 h. **A 3-4-fold maximum increase in levels of immunoreactive AM protein was detected after 6-8 h of stimulation**. While LPS-hyporesponsive C3H/HeJ macrophages failed to respond to protein-free LPS with an increase in AM steady-state mRNA levels, increased levels were observed after stimulation of these cells with a protein-rich (butanol-extracted) LPS preparation. In addition, increased AM mRNA was observed following treatment of either C3H/OuJ or C3H/HeJ macrophages with soluble *T. gondii* tachyzoite antigen (STAg) or the synthetic flavone analog 5,6-dimethylxanthenone-4-acetic acid (5,6-MeXAA). Interferon-γ (IFN-γ) also stimulated C3H/OuJ macrophages to express increased AM mRNA levels, **yet was inhibitory in the presence of LPS or Taxol**. *In vivo*, mice challenged i.p. with 25 μg LPS exhibited increased AM mRNA levels in lung, liver and spleen, with the greatest increase (>50-fold) observed in the liver and lung. Thus, AM is produced by murine macrophages, and furthermore, LPS induces AM mRNA *in vivo* in a number of tissues. These data support a possible role for AM in the pathophysiology of sepsis and septic shock.

INTRODUCTION

LPS is a potent inflammatory stimulus derived from the outer membrane of Gram negative bacteria. Release of LPS from dying bacteria can initiate a serious systemic inflammatory response to infection, resulting in septic shock. Septic shock is typified by fever, hypoglycemia, hypotension, disseminated intravascular coagulation, multi-organ failure, and shock that may result in death (5, 33, 34). Septic shock continues to have an associated mortality rate of 40 to 70% and remains the leading cause of death in intensive care units (1, 33, 34). The interaction of LPS with host cells initiates the production of a cascade of proinflammatory mediators that are responsible for its effects (25). The release of cytokines like tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-12, interferon-γ (IFN-γ), nitric oxide ($NO^{\cdot}$), and colony stimulating factor from monocytes and macrophages elicit the physiologic changes observed during sepsis and septic shock (25, 30, 34, 40). The antitumor agent, Taxol, is a LPS-mimetic in murine macrophages. Shared activities include the ability to activate murine macrophages to express a wide variety of inflammatory and anti-inflammatory genes, tyrosine phosphorylate MAP kinases, secrete cytokines, induce translocation of NF-κB and upregulate autophosphorylation of *Lyn* kinase. In addition, Taxol provides a second signal to IFN-γ-primed murine macrophages to become tumoricidal and to produce nitric oxide (8, 11, 22, 24, 36). Macrophage responsiveness to LPS and Taxol are both linked to the *Lps* gene. The C3H/HeJ mouse strain expresses a defective allele at this locus and macrophages derived from this mouse strain are hyporesponsive not only to LPS (45) but also to Taxol (22, 24).

AM is a hypotension-causing peptide that was originally isolated from human pheochromocytoma cells (19). It induces vasorelaxation that leads to a persistent depression of blood pressure (15). In previous studies, AM mRNA was found to be expressed in various organs, including the cardiovascular system, lung, adrenal gland,

cultured endothelial cells, vascular smooth muscle cells, alveolar and endometrical macrophages, and in virtually all tumor cell lines examined (19, 27, 29, 38, 43, 44, 48). **Moreover, AM was recently demonstrated to exhibit direct antimicrobial activity (46).** The concentration of AM in plasma is increased in patients with hypertension, septic shock, and heart failure, suggesting that AM may participate in the regulation of blood pressure and contribute to refractory hypotension in septic shock (14, 18). Given the plethora of bioactive peptides released by LPS-activated macrophages, we postulated that AM may also be produced by macrophages in response to LPS as a result of Gram negative infection, and perhaps, contribute to the hypotension associated with Gram negative sepsis and septic shock. In the present study, we demonstrated that LPS and Taxol, as well as other potent macrophage stimuli, induce AM mRNA **and protein** expression in murine peritoneal macrophages. Additionally, AM mRNA levels were upregulated in the lung, liver, and spleen following LPS injection.

MATERIALS AND METHODS

Reagents. Phenol-water-extracted *E. coli* K235 LPS (PW-LPS ; <0.008% protein) was prepared by the method of McIntire et al. (28). Protein-rich, butanol-extracted *E.coli* K235 LPS (But-LPS; ~18% protein) was prepared as described by Morrison and Leive (31). Taxol was kindly provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and was stored at -70° C as a 20 mM stock solution in dimethylsulfoxide. A 1 mM stock of Taxol contained <0.03 endotoxin U/ml by the *Limulus* amoebocyte lysate assay. 5,6-MeXAA was synthesized by the Cancer Research Laboratory, University of Auckland, Auckland, New Zealand (2, 37). A stock solution of 5,6-MeXAA was freshly prepared for each experiment by solubilizing the compound in sterile endotoxin-free 5% $NaHCO_3$ by vortexing. Once solubilized, the solution was diluted in supplemented RPMI 1640 medium containing 2% FCS to obtain a 10 mg/ml stock solution that was then diluted to the required concentration for macrophage stimulation. The endotoxin level of the highest concentration of 5,6-MeXAA used in these experiments was <0.0125 ng/ml as detected by *Limulus* amoebocyte lysate assay. A soluble extract of *T. gondii* tachyzoites (STAg) was a gift from Dr. Alan Sher, NIAID, NIH. Recombinant murine IFN-γ (1.3×10^7 U/ml) was kindly provided by Genentech, Inc. (South San Francisco, CA). Cyclohexamide (CHX) was obtained from Sigma Chemical co. (St Louis, MO) and used at a final concentration of 5 μg/ml.

Mice. For *in vivo* analysis of AM gene induction, 6-8 wk old C57BL/6J mice were injected i.p. with 25 μg of LPS. Four mice were used per time point per treatment. GKO mice were a gift from Genentech, Inc. (San Francisco, CA) (7).

Macrophage isolation and cell culture conditions. Five to 6-week old female C3H/OuJ and C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine), maintained in a laminar flow facility under 12 h alternating light-dark

cycles, and fed standard laboratory chow and acid water *ad libitum*. Research was conducted according to the principles set forth in "Guide for the Care and Use of Laboratory Animals". Mice were injected i.p. with 3 ml of 3% fluid thioglycollate. Four days later, peritoneal exudate cells were extracted by peritoneal lavage. Cells were washed once and resuspended in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin per ml, 10 mM HEPES, 0.3% sodium bicarbonate, 2% fetal calf serum, and added to six-well tissue culture plates (Falcon, Lincoln Park, NJ) at ~4.0 x 10^6 cells per well in a final volume of 2.0 ml. The plates were incubated at 37° C and 6% CO_2. After a 12 h adherence period, nonadherent cells were washed off and the adherent macrophages were treated with 2.0 ml of medium or medium containing the indicated substances. **For the detection of AM in culture supernatants, macrophages were cultured at ~6 x 10^6 cells per well in six-well tissue culture plates in a total volume of 3.0 ml and supernatants collected at the indicated times after stimulation with LPS or Taxol.**

Isolation of total cellular RNA. For *in vitro* experiments, Culture supernatants were removed, and the cells were solubilized in 1 ml of RNA Stat60 (Tel-Test 'B', Inc., Friendswood, TX). For *in vivo* experiments, the liver, lung and spleen were removed from individual mice and frozen at -70° C. Tissues were homogenized in RNA Stat60. Total cellular RNA was extracted from *in vitro* and *in vivo* samples according to the manufacturer's instructions and quantified by spectrophotometric analysis.

Analysis of tissue mRNA by reverse transcription (RT)-PCR. Relative quantities of mRNA for hypoxanthine-guanine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and AM were determined by a coupled RT-PCR as detailed elsewhere (9). For the AM gene, the following oligonucleotide sequences were used:

Sense: 5'-AAGAAGTGGAATAAGTGGGCG

Antisense: 5'-ACCAGATCTACCAGCTAACA

Probe: 5'-CCCCCTACAAGCCAGCAATCAG.

Primer sequences for the detection of AM mRNA were chosen by analysis of the murine genomic sequence and amplify a 284 bp product. The probe sequence for AM was chosen in conjunction with the published murine cDNA sequences obtained from GenBank. Primer and probe sequences for the housekeeping genes, HPRT or GAPDH have been reported (3). The PCR annealing temperatures were 54° C, 55° C and 54° C for HPRT, GAPDH and AM, respectively. The PCR cycle numbers were 28 and 35 for *in vitro* and *in vivo* AM induction, respectively. The PCR cycle number for HPRT and GAPDH were 24 in both cases. Amplified products were analyzed by electrophoresis followed by Southern blotting and hybridization with the nonradioactive internal oligonucleotide probe. Chemiluminescent signals were quantified using a scanning densitometer (Datacopy GS plus, Xerox Imaging Systems, Sunnyvale, CA). To determine the magnitude of change in gene expression, cDNA from a sample known to be positive for AM and HPRT or GAPDH were used to generate standard curves by serial 2-fold dilution of the positive control and simultaneous amplification. The signal of each band in the standard curve was plotted and subjected to linear regression analysis. The equation from this line was used to calculate the fold induction in test samples. Results were normalized for the relative quantity of mRNA by comparison to HPRT or GAPDH. In each *in vitro* experiment, means are expressed relative to medium controls. *In vivo*, means are expressed relative to saline injected controls (t = 0) which were assigned a value of 1.

Detection of AM in macrophage culture supernatants. Levels of immunoreactive AM were detected in macrophage culture supernatants by radioimmunoassay (RIA) as described previously (26).

Statistics. Results were analyzed using Student's *t* test for comparisons between two groups.

RESULTS

AM mRNA and protein induced by PW-LPS or Taxol in murine macrophages. Previous studies have demonstrated that LPS induces AM mRNA **and protein** expression in cultured rat aortic vascular smooth muscle cells (42) and in cultured endothelial cells (41). In the present study, we investigated if AM mRNA expression **and protein secretion** were modulated in murine macrophages by PW-LPS or by the LPS-mimetic, Taxol. Endotoxin-responsive C3H/OuJ macrophages were treated for 1, 2, 4, 6, 8, and 24 h with medium alone, 100 ng/ml of PW-LPS, or 35 μM Taxol. RNA was isolated and AM and HPRT mRNA were detected by RT-PCR. As shown in Figure 1A, the kinetics of AM gene induction by PW-LPS and Taxol were remarkably similar, with AM mRNA expression being induced by PW-LPS or Taxol as early as 1 h, peaking at 2 h (>10-fold over baseline), and gradually returning to basal levels by 24 h. To assess the sensitivity of AM mRNA to induction by PW-LPS or Taxol, dose response analyses were performed (Figure 1B). Murine C3H/OuJ peritoneal macrophages were treated for 2 h, the time when AM mRNA expression had peaked, with various concentrations of PW-LPS or Taxol. RNA was isolated and AM and HPRT mRNA was detected by RT-PCR. As little as 0.1 ng/ml PW-LPS induced AM mRNA expression (>2-fold), while ≥10 ng/ml of PW-LPS was necessary to induce maximal (>10-fold) AM mRNA expression. A comparable increase in AM gene expression was induced by 5 to 35 μM Taxol. **Macrophage culture supernatants were also analyzed by RIA for the presence of immunoreactive AM. Figure 1C illustrates that both LPS and Taxol induce AM secretion several hours after the appearance of AM mRNA, with a maximal induction of 3-4-fold over basal levels.**

PW-LPS- or Taxol-induced AM mRNA requires a normal *Lps* gene product. Previous studies in our laboratory have demonstrated the induction of an extensive panel of inflammatory genes (*e.g.*, TNFα, IL-1β, TNF receptor type II,

interferon-inducible-protein-10, D3, and D8) by various LPS preparations or Taxol in macrophages derived from LPS-responsive (*Lps*^{n}) C3H/OuJ mouse strain (22, 45). In contrast, macrophages derived from LPS-hyporesponsive (*Lps*^{d}) C3H/HeJ mouse strain failed to express any of the above genes in response to either PW-LPS or Taxol. Despite their inability to respond to PW-LPS, C3H/HeJ macrophages are responsive to LPS extracted with butanol (But-LPS), a milder extraction process in which LPS remains associated with membrane proteins. Moreover, C3H/HeJ and C3H/OuJ macrophages exhibit comparable sensitivity to endotoxin-associated proteins isolated from protein-rich LPS preparations (12) and to a soluble antigen extract of *T. gondii* tachyzoites (STAg). Both protein-rich LPS and STAg result in tyrosine phosphorylation of MAPK and induced a subset of LPS-regulated genes (21). The anti-tumor agent, 5,6-MeXAA, is also active on both C3H/HeJ and C3H/OuJ macrophages (35). Therefore, we next investigated whether any of these agents would induce AM gene expression in C3H/HeJ macrophages. Peritoneal macrophages from C3H/HeJ mice were treated with medium alone, 50 μg/ml STAg, 10 μg/ml 5,6-MeXAA, 10 μg/ml But-LPS, 100 ng/ml PW-LPS, or 35 μM Taxol for 2 or 4 h. These concentrations were chosen based on optimal induction of gene expression by these agents in previous studies (12, 21, 35). RNA was isolated and AM and HPRT mRNA levels were quantified. At 2 h, only STAg and But-LPS, but not 5,6-MexAA, had significantly increased AM gene expression (≥6 fold; data not shown). As expected, neither PW-LPS nor Taxol induced AM mRNA in C3H/HeJ macrophages (Figure 2). By 4 h, STAg , But-LPS, and 5,6-MeXAA, had increased AM gene expression in C3H/HeJ macrophages >4-6-fold over baseline (Figure 2). LPS-responsive macrophages from C3H/OuJ mice also responded to PW-LPS, Taxol, STAg, or 5,6-MeXAA to express heightened levels of AM mRNA (>10 fold) (data not shown). These data indicate that although the *Lps*^{d} allele precludes induction of AM mRNA by PW-LPS or Taxol, these cells respond to STAg, 5,6-MeXAA, and But-LPS with increased expression of AM mRNA.

Expression of AM mRNA in macrophages treated with cycloheximide . To determine whether induction of AM mRNA by LPS or Taxol requires *de novo* protein synthesis, C3H/OuJ macrophages were treated for 2 h with medium, 100 ng/ml PW-LPS, or 35 μM Taxol, in the absence or presence of 5 μg/ml of the protein synthesis inhibitor, cycloheximide (CHX). This concentration of CHX has been shown previously to inhibit expression of other LPS-inducible genes in C3H/OuJ macrophages (3). RNA was isolated and both AM and GAPDH mRNA were detected by RT-PCR (Figure 3). CHX alone induced accumulation of steady-state AM mRNA. In addition, higher levels of steady-state AM mRNA were observed after macrophages were treated for 2 h with either PW-LPS and CHX or with Taxol and CHX (Figure 3). Thus, accumulation of AM mRNA is not dependent on *de novo* protein synthesis.

IFN-γ upregulates AM mRNA expression and negatively regulates LPS- and Taxol-induced AM mRNA expression. We next assessed the ability of a second potent macrophage activating agent, IFN-γ, to regulate AM mRNA expression. C3H/OuJ peritoneal macrophages were treated with IFN-γ (5 U/ml) for 2, 4, 6, and 24 h. RNA was isolated and AM and HPRT mRNA were detected by RT-PCR. As shown in Figure 4A, AM mRNA expression was induced by IFN-γ as early as 2 h and peaked at 4 to 6 h (>7-fold), and returned to basal levels by 24 h. In many instances, IFN-γ provides a "priming" signal that results in the synergistic induction of gene expression and secreted products (*e.g.*, TNFα, NO·, IL-6) when provided with a second triggering signal such as LPS (13, 47). To ascertain whether IFN-γ would modulate in the induction of AM by LPS, C3H/OuJ macrophages were cultured for 4 h with PW-LPS in the absence or presence of IFN-γ. As shown in Figure 4B, IFN-γ (5 U/ml) down-regulated LPS-induced AM mRNA levels. Similar results were observed when the macrophages were stimulated simultaneously with both Taxol and IFN-γ (5 U/ml) (**Figure 4C**).

LPS-induced AM mRNA *in vivo*. Previous studies from this laboratory have demonstrated that LPS elicits gene expression *in vivo* that is both organ- and gene-specific (39, 40). To assess whether LPS augments AM mRNA levels *in vivo*, C57BL/6 mice were challenged i.p. with 25 μg of LPS and AM mRNA expression was assessed in liver, lung, and spleen. As shown in Figure 5, AM mRNA expression was rapidly induced (by 1 h) in the liver. Hepatic AM mRNA remained at heightened levels (~20-60 fold above baseline) from 3 to 8 h after LPS challenge, then returned to near basal levels by 12 h. In contrast, increased AM mRNA expression was not observed in the lung until 6-8 h after LPS challenge and pulmonary AM mRNA peaked after 12 to 24 h (~50 fold). In contrast to both the liver and the lung, splenic AM mRNA expression was poorly modulated (~4-fold, in 3 h) by LPS and by 24 h, splenic AM mRNA expression was substantially downregulated (~10-fold below basal AM mRNA levels).

Endogenous IFN-γ regulates LPS-induced AM mRNA *in vivo*. *In vitro*, IFN-γ suppressed LPS-induced AM mRNA in C3H/OuJ macrophages (Figures 4B). To examine the role of IFN-γ in the *in vivo* regulation of AM mRNA by LPS, mice with a targeted disruption in the IFN-γ gene (GKO) (7) were utilized. Basal hepatic AM mRNA was ~4-fold higher in the liver of GKO than C57BL/6 mice (Figure 6). Interestingly, no increase in hepatic AM mRNA was observed after LPS challenge. In fact, by 6 h following LPS administration, AM mRNA levels had returned to baseline levels exhibited by the control C57BL/6 mice.

DISCUSSION

LPS, the endotoxic outer membrane component of Gram negative bacteria, has long been implicated in the pathophysiology of septic shock. The inflammatory syndrome that is associated with sepsis is characterized by hypotension and multiple organ dysfunction, which is felt to be initiated by the action of secondary inflammatory mediators released from LPS-stimulated cells. The systemic inflammatory response induced by LPS or Gram negative bacterial infection can be partially ameliorated by blocking either LPS itself or downstream endogenous mediators, such as IL-1 and TNF-α. Many of these mediators are produced by macrophages. However, in the clinical setting, blocking LPS itself is of limited value since the deleterious effects have already been initiated by the time the inflammatory syndrome is apparent (49). Based on preclinical data, the approach of blocking endogenous inflammatory mediators, such as TNF-α, seemed promising, however, clinical trials have yet to demonstrate clear efficacy using this approach in septic shock (32). AM, originally identified in pheochromocytoma, is a ubiquitously expressed peptide that is a member of the calcitonin-related peptide superfamily (19). It possesses both potent vasodepressor (15, 19) and cardiodepressor (20, 38, 43) activities, and increased plasma AM levels have been reported in a variety of clinical conditions associated with blood pressure and hemodynamic alterations, suggesting that it participates in blood pressure regulation (14, 18). While LPS had been shown previously to induce AM gene transcription in endothelial and vascular smooth muscle cells (41), it was not known whether LPS also induced AM in macrophages. The data presented herein demonstrate that LPS causes a rapid induction in AM gene transcription in peritoneal macrophages *in vitro*, peaking within 2 hours and gradually subsiding within 24 hours, a kinetic profile very similar to that of other LPS-inducible, proinflammatory genes (21, 22, 35). Furthermore, AM gene induction is not dependent upon new protein synthesis, implying the existence of a preformed signal transduction apparatus. Moreover, the finding that CHX alone increased AM steady-state mRNA levels suggests that AM gene expression may be

maintained in a suppressed state due to the action of a CHX-sensitive suppresion molecule. **The increase in AM mRNA was followed by the secretion of immunoreactive AM into the culture supernatants.** Thus, our study lends support to the notion that AM, like TNF-α and IL-1, might serve as an endogenous mediator of the inflammatory syndrome associated with sepsis. This remains to be proven by blocking its action *in vivo*; however, blocking anti-AM antibodies are not currently available. **More recently, AM has been shown to be directly microbicidal (46), suggesting the possibility that AM, like other cytokines and chemokines, might be stimulated by bacterial products, such as LPS, as a normal part of the macrophage's innate response to infection. This hypothesis is strengthened by our findings that two other bacterial stimulants, STAg and endotoxin-associated proteins, were also found to be potent stimuli (Fig. 2).**

It is also known that AM significantly enhanced $NO^{\cdot}$ synthesis evoked by LPS and IFN-γ in cultured vascular smooth muscle cells. Thus, AM may contribute to circulatory failure during endotoxin shock, in part, by modulating $NO^{\cdot}$ release (42). **However, we were unable to activate C3H/OuJ macrophages with synthetic AM (up to 1 mM) to release $NO^{\cdot}$, and the presence of synthetic AM failed to modulate $NO^{\cdot}$ release stimulated by LPS and/or IFN-γ (data not shown). Thus, it appears that the induction of these two vasodilatory substances by LPS are regulated independently.**

We have previously shown that the anti-tumor, chemotherapeutic agent, Taxol, mimics the effects of LPS on murine macrophages. Both are dependent upon the expression of a normal *Lps* allele, are blocked by the same LPS analog antagonists, cause tyrosine phosphorylation of MAP kinases and autophosphorylation of *lyn* kinase, induce translocation of NF-κB, and induce an indistinguishable pattern of cytokine gene expression and secretion (6, 25) . Furthermore, the effect of Taxol appears to be

independent of its well characterized microtubule-binding activity, as evidenced by the failure of Taxol analogs with varying microtubule binding capacities to correlate with LPS-mimetic activity (17, 25, 45). The data presented herein demonstrates that like LPS, AM is induced in murine macrophages by Taxol. Interestingly, Taxol causes hypotension in ~10% of patients within 3 h of administration and the mechanism for this side effect is unknown, but is consistent with the possibility that Taxol-induced AM may be a contributing endogenous mediator. AM has recently been shown to act as a local autocrine growth factor in a variety of human tumors (29). The anti-tumor activity of Taxol is believed to be due primarily to its anti-mitotic activity on tumor cells, although it has also been shown to activate tumoricidal macrophages (4, 24) and to inhibit angiogenesis. The role of Taxol-induced production of AM by neoplastic cells will be the focus of future studies.

In vivo, LPS induced an almost 100-fold increase in AM gene expression in the liver, reaching peak expression within a few hours, whereas later induction was observed in the lung, with fundamentally no AM induction in the spleen. LPS has been shown to cause elevated AM levels in plasma of rat aortic vascular smooth muscle cells, and in endothelial cells tissue from anesthetized rats (42), as well as to induce 2-3 fold increase in AM mRNA levels in a variety of organs including lung and intestine (41). Plasma levels reflect both local hemodynamic distribution, as well as production and secretion. Thus studying protein levels may not be an accurate measure of secretion by a given organ. By studying the time course of gene induction directly, we could localize the liver as a major site of early AM production in response to LPS, with kinetics common to those of acute phase reactants. The particular hepatic cell type (*i.e.*, resident histiocytic Kuppfer cells, hepatocytes, or others) that is responsible for this increase remains to be elucidated, as is the relative contribution of other parenchymatous tissues not examined here.

The possible role of IFN-γ in the regulation of AM gene expression is another novel aspect of this study. IFN-γ alone is nearly as potent an inducer of AM gene

expression as LPS *in vitro*, yet in contrast to many LPS-inducible genes, where IFN-γ and LPS synergize (e.g., TNF-α , iNOS, etc.) (13, 47), AM gene expression was antagonized when both IFN-γ and LPS **or Taxol** were present simultaneously. This pattern of mitigated gene expression in the presence of both IFN-γ and LPS has been reported for several other LPS-inducible genes, including KC, IL-1β, Type II TNF receptor, and Secretory Leukocyte Protease Inhibitor (10, 16, 23). At this juncture, the molecular interaction that result in this antagonism is not yet understood. *In vivo*, IFN-γ has been implicated as a critical cytokine in LPS-induced toxicity (39). Mice with a targeted mutation of the IFN-γ gene (*i.e.*, GKO mice) exhibited elevated basal AM expression that was down-regulated only after LPS injection. These data support the hypothesis that IFN-γ must necessarily interact with some additional LPS-inducible inflammatory mediator to maintain AM levels in the normal mouse.

Taken collectively, our data suggest that AM may be viewed additional immediate early gene produced predominantly by liver in response to LPS. The relative contribution of AM to hypotension and septic shock remains to be elucidated by blocking its secretion or action.

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FIGURE LEGENDS

Figure 1. PW-LPS and Taxol induce AM mRNA **and protein synthesis** in C3H/OuJ macrophages. A. Kinetics of PW-LPS- and Taxol- induced AM mRNA expression. C3H/OuJ macrophages were cultured for the indicated time with medium, 100 ng/ml PW-LPS, or 35 μM Taxol. mRNA was isolated and AM and HPRT mRNA detected by RT-PCR. Data represent the arithmetic mean ± SEM (n=7 separate experiments). B. Dose-dependent induction of AM mRNA. C3H/OuJ macrophages were cultured for 2 h with medium or with the indicated concentrations of PW-LPS or Taxol. mRNA was isolated and AM and HPRT mRNA detected by RT-PCR. Data represent the arithmetic mean ± SEM (n=4). **C. Kinetics of PW-LPS and Taxol-induced AM secretion. C3H/OuJ macrophages were cultured for the indicated time with medium, 100 ng/ml LPS, or 35 μM Taxol. Macrophage culture supernatants were analyzed by RIA for the presence of immunoreactive AM. Data were derived from a representative experiment (n = 3).** When not visible, SEM bars are smaller than the symbol.

Figure 2. Neither PW-LPS nor Taxol induced AM gene expression in LPS-hyporesponsive C3H/HeJ macrophages *in vitro.* C3H/HeJ macrophages were treated for 4 h with medium, 5 μg/ml STAg, 10 μg/ml MeXAA, 5 μg/ml But-LPS, 100 ng/ml PW-LPS, or 35 μM Taxol. Data represent the arithmetic mean ± SEM (n=4). When not visible, SEM bars are smaller than the symbol.

Figure 3. *De novo* protein synthesis is not required for PW-LPS- or Taxol-induced AM mRNA production. C3H/OuJ macrophages were treated for 2 h with either medium, 100 ng/ml PW-LPS, 5 μg/ml CHX, both 100 ng/ml PW-LPS and 5 μg/ml CHX, 35 μM Taxol, or both 35 μM Taxol and 5 μg/ml CHX. RNA was

isolated and AM and GAPDH mRNA were detected by RT-PCR. A representative Southern blot is shown (n=3).

Figure 4. Regulation of AM mRNA expression by IFN-γ in C3H/OuJ macrophages. A. IFN-γ upregulates AM mRNA expression. C3H/OuJ macrophages were cultured for the indicated times with medium or 5 U/ml IFN-γ. Data are derived from a representative experiment. B. IFN-γ down-regulates PW-LPS-induced AM mRNA expression. C3H/OuJ macrophages were cultured for 4 h in the presence of medium only or increasing concentrations of PW-LPS in the absence or presence of 5 U/ml IFN-γ. A representative Southern blot is shown (n=3). **C. IFN-γ down-regulates Taxol-induced AM mRNA expression. C3H/OuJ macrophages were cultured for 4 h in the presence of medium only, 5 μM Taxol, or 35 μM Taxol, in the absence or presence of 5 U/ml IFN-γ. Data represent the arithmetic mean ± SEM of 3 separate experiments.**

Figure 5. LPS augments AM mRNA expression *in vivo*. C57BL/6 mice were injected i.p. with 25 μg of LPS. Data are the mean fold increase in AM mRNA expression ± SEM from 4 to 8 individual mice at each time point. Means are expressed relative to the saline injected control value (t=0), which is arbitrarily assigned a value of 1. When not visible, SEM bars are smaller then the symbol.

Figure 6. Endogenous IFN-γ regulates LPS-induced AM mRNA *in vivo*. GKO and C57BL/6 mice were injected i.p. with 25 μg of LPS and LPS-induced AM mRNA was quantified in the liver. Data are expressed as the arithmetic mean ± SEM of values from 5 to 8 mice at each time point.

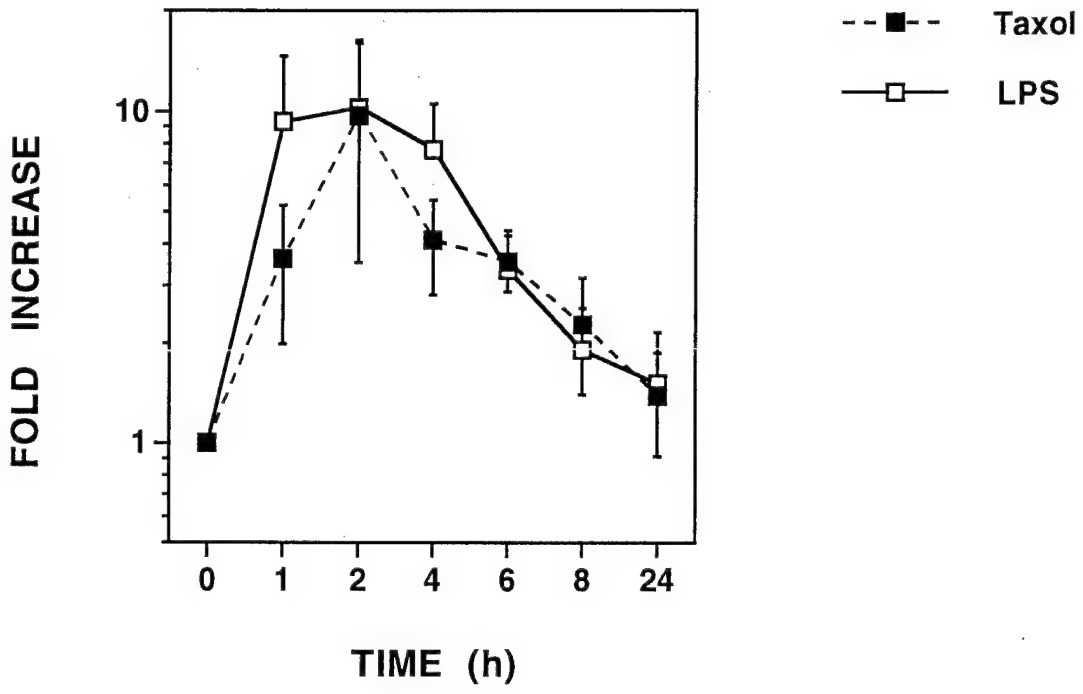

FIGURE 1A

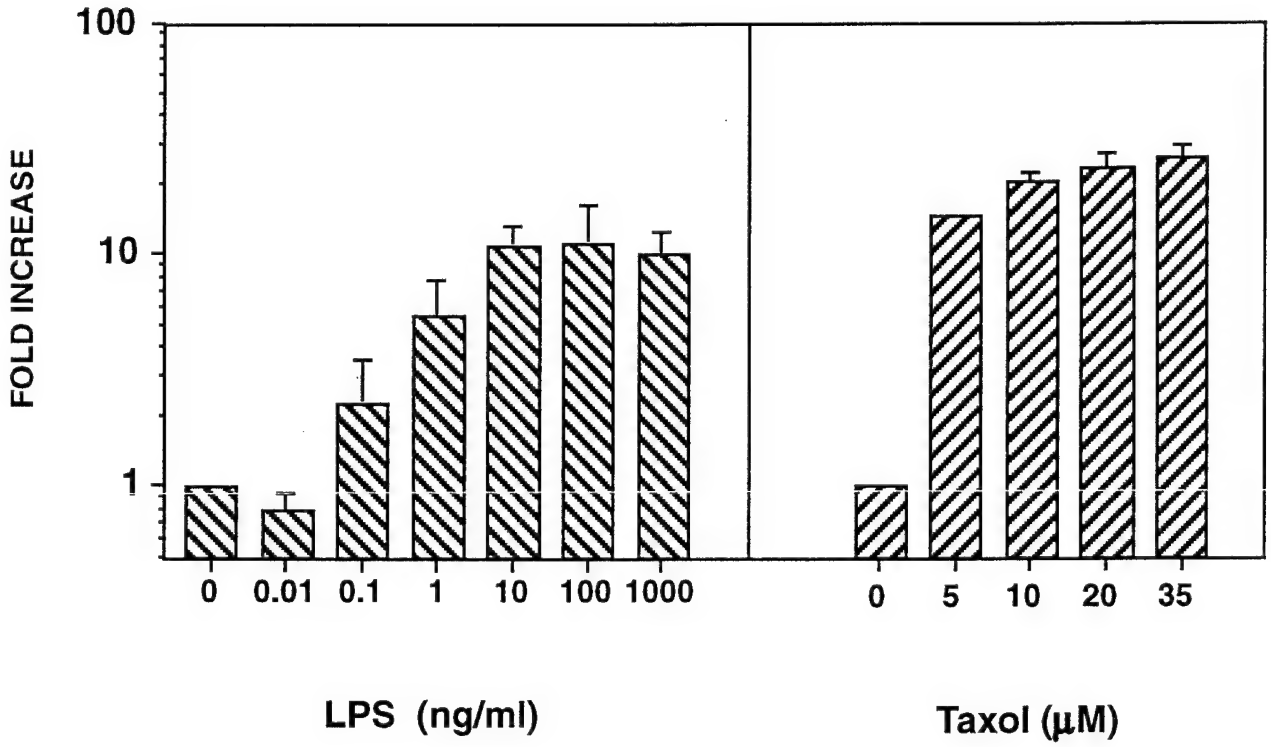
100
10
1
FOLD INCREASE
0
0.01
0.1
1
10
100
1000
LPS (ng/ml)
0
5
10
20
35
Taxol (μM)

Figure 1B

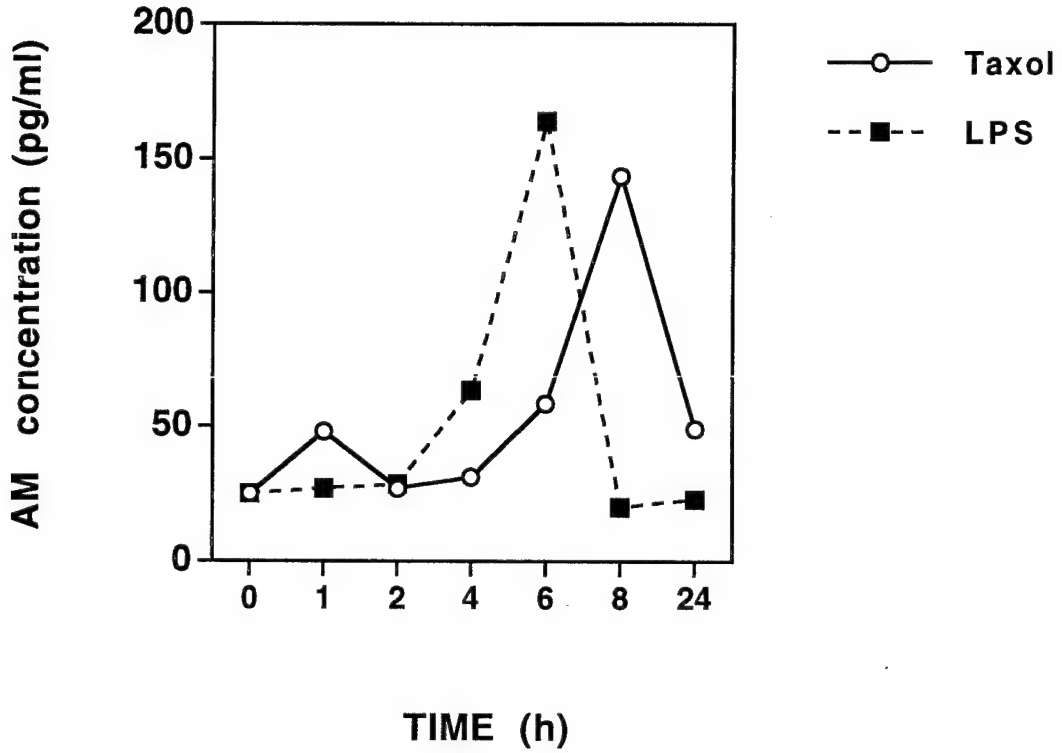
AM concentration (pg/ml)
200
150
100
50
0
0
1
2
4
6
8
24
TIME (h)
Taxol
LPS

FIGURE 1C

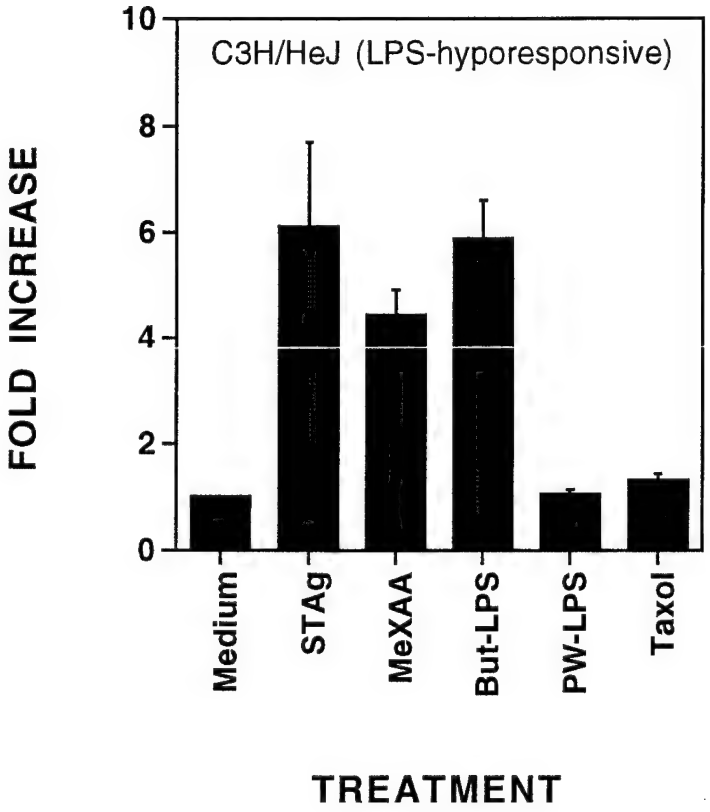
C3H/HeJ (LPS-hyporesponsive)
FOLD INCREASE
10
8
6
4
2
0
Medium
STAg
MeXAA
But-LPS
PW-LPS
Taxol
TREATMENT

FIGURE 2

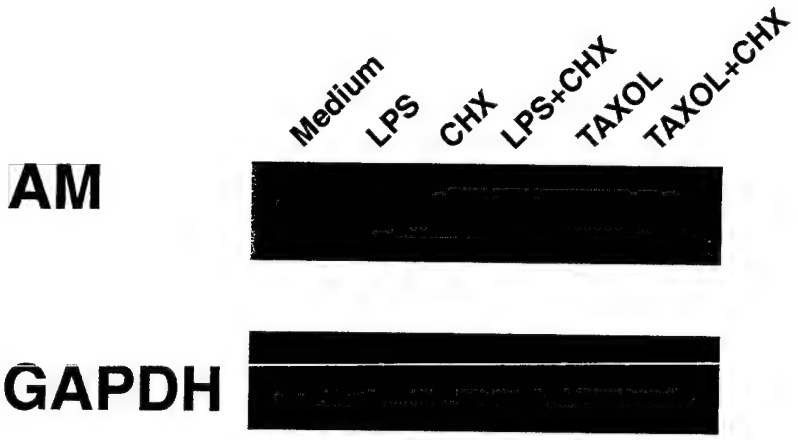

Figure 3

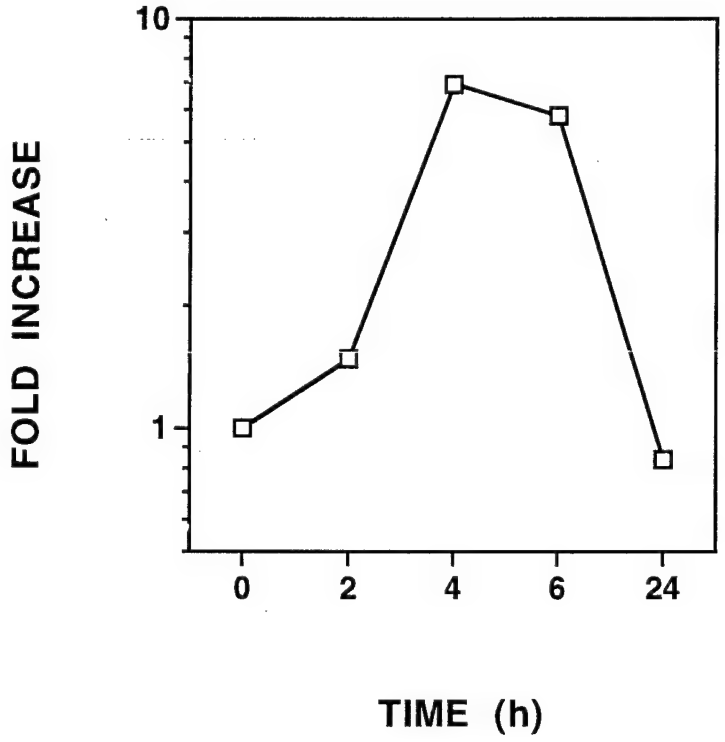

Figure 4A

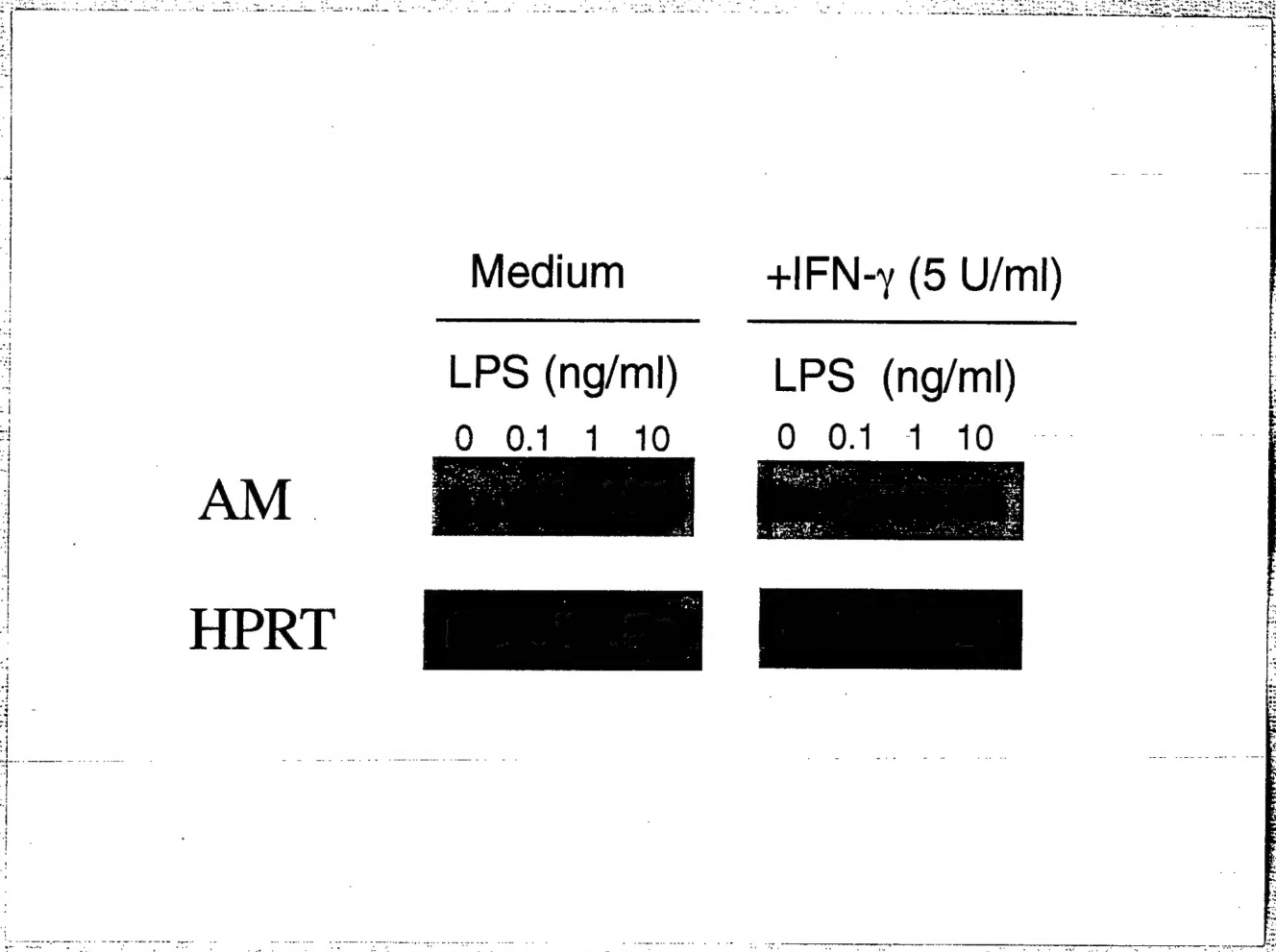

Figure 4B

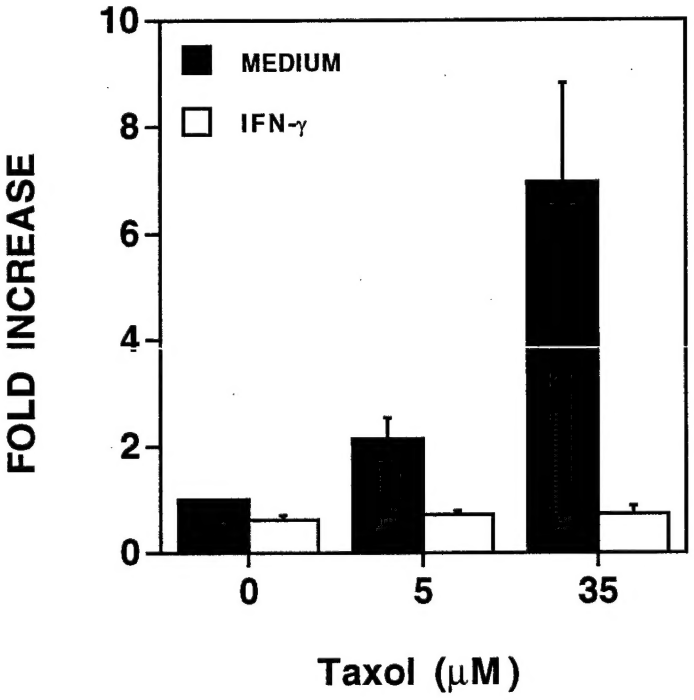

Figure 4C

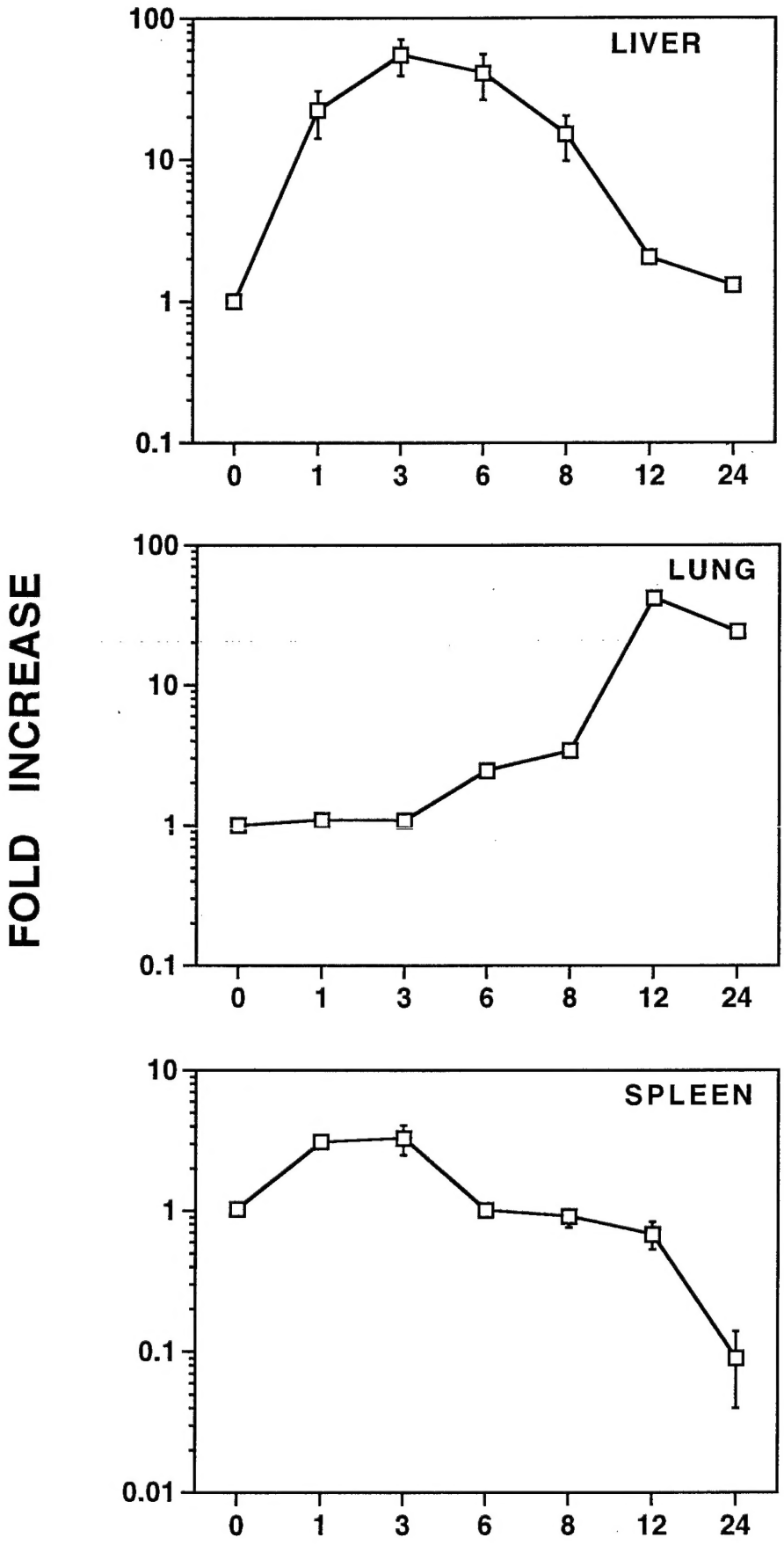
LIVER
LUNG
SPLEEN
100
10
1
0.1
0.01
0
1
3
6
8
12
24
FOLD INCREASE
TIME (h)

Figure 5

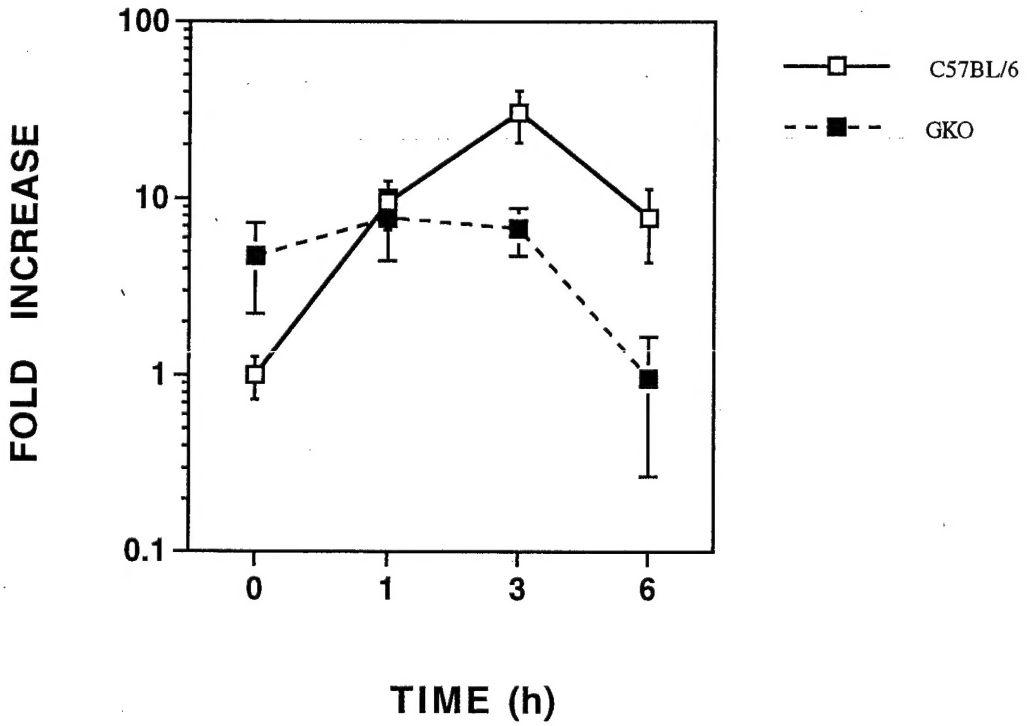

Figure 6